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(54) Title: T-CELLS SPECIFICALLY RECOGNIZING MINOR HISTOCOMPATIBILITY ANTIGEN(S) AND USES THEREOF FOR ELIMINATING TARGET CELLS

(57) Abstract: This invention relates to T-cells that specifically recognize minor histocompatibility antigen(s), methods for selecting these T-cells and uses thereof for eliminating target cells, and more particularly for eliminating hematopoietic cancerous cells. The invention is based on 1) the priming of T-cells specifically reacting against a selected immunodominant ubiquitous MiHA that is expressed by target cells and by non-target cells; and also 2) the selection of a 100 % purified population of T-cells that specifically react against an immunodominant minor histocompatibility antigen which is ubiquitously expressed by the recipient's cells or selectively expressed by specific recipient's target cells only. The major advantage of the invention is that the T-cells used therein can be transferred from a donor to a compatible recipient without causing to the latter a graft-versus-host disease (GVHD) reaction.

T-CELLS SPECIFICALLY RECOGNIZING MINOR HISTOCOMPATIBILITY ANTIGEN(S) AND USES THEREOF FOR ELIMINATING TARGET CELLS

BACKGROUND OF THE INVENTION

a) Field of the invention

The present invention is concerned with the elimination of undesirable target cells such as cancerous cells, viral infected cells and abnormal cells of a recipient. More particularly, the invention is concerned with the isolation, selection and use of T-cells from a donor for the treatment of hematopoietic cancers.

b) Brief description of the prior art

Adoptive immunotherapy is a main approach that is currently being investigated in the field of cancer immunotherapy. Adoptive immunotherapy is based on the use of T-cells specifically recognizing minor histocompatibility antigens (MiHAs). According to this approach, patients with hematological malignancies are treated by allogeneic hematopoietic cell transplant (AHCT) from a hematopoietic cancer-free donor. Following AHCT, eradication of leukemic cells is primarily mediated by a donor T-cell dependent immune reaction commonly referred to as the graft-versus-leukemia (GVL) effect.

However, toxicity is one major impediment to MiHA-based adoptive immunotherapy. The GVL effect is indeed commonly associated with a graft-versus-host-disease (GVHD) reaction of the recipient, a reaction that is lethal in many cases. The potentially fatal condition of GVHD has also greatly limited the use of MiHA-based adoptive immunotherapy to hematopoietic cancer treatments only, although this approach could, in theory, be used for treating other types of cancers and other diseases such as chronic viral infections and abnormal cells proliferation.

To reduce the risks of GVHD when using the approach of MiHA-based adoptive immunotherapy for treating hematopoietic-cancerous recipients, International PCT patent application published under No. WO 99/05173 has proposed a method wherein CD8+ T-cells from a hematopoietic cancer-free donor

are i) isolated; ii) primed against a minor histocompatibility antigen (MiHA) selectively expressed by hematopoietic cells; and iii) transferred into a hematopoietic-cancerous recipient. However, the feasibility of the approach proposed in WO 99/05173 has not been demonstrated in a way as to eradicate cancer cells of a recipient without causing him a GVHD. Furthermore, the main problem with the approach proposed in WO 99/05173 is that it is generally complicated to find MiHAs expressed solely by hematopoietic cells.

There is thus a need for T-cells that could be used in adoptive immunotherapy methods without causing the recipient a graft-versus-host disease (GVDH) reaction. Also needed are T-cells that would allow to eradicate not only hematopoietic cancerous cells of a recipient, but also other types of cancerous cells, viral infected cells and abnormal cells. There is also a need for methods for selecting such T-cells and for therapeutic physiological solutions comprising the same.

There is further a need for adoptive immunotherapy methods wherein ubiquitous minor histocompatibility antigens (MiHAs) are targeted, i.e. minor histocompatibility antigens that are expressed not only by target cells to be eliminated but also expressed by non-target cells. There is more particularly a need for methods for treating an hematopoietic cancerous recipient without causing him a graft-versus-host disease (GVHD) reaction.

The present invention fulfils these needs, since it demonstrates for the first time that MiHA based immunotherapy can cure leukemia without causing GVHD. The invention fulfils also other needs which will be apparent to those skilled in the art upon reading the following specification.

SUMMARY OF THE INVENTION

In accordance with the present invention, T-cells, method for selecting the same, therapeutic physiological solutions and methods of treatment, are provided that are effective for eliminating target cells in a patient without causing him a GVDH reaction. The invention is more particularly concerned with the treatment of hematopoietic cancers.

In one aspect, the invention is directed to a population of T-cells that have been isolated from a donor and that have been selected for specifically recognizing an immunodominant minor histocompatibility antigen (MiHA) expressed by target cells of a recipient but not expressed by any of the donor's cells. At least some of the T-cells from the population of T-cells are capable of being primed against the immunodominant MiHA such that, when transferred into a recipient, the primed T-cells eliminate the recipient's target cells without causing him a graft-versus-host disease (GVHD) reaction. In a preferred embodiment, the T-cells population comprises a plurality of CD8+ T-cells that have been isolated from a hematopoietic cancer-free donor and that have been selected for specifically recognizing a ubiquitous and immunodominant minor histocompatibility antigen (MiHA) expressed by hematopoietic cancerous cells of a recipient, but not expressed by any of the donor's cells.

In another aspect, the invention is directed to the use of population(s) of the above-mentioned T-cells for the elimination of target cells, such as hematopoietic cancerous cells in a recipient and to therapeutic physiological solutions comprising such population(s) of T-cells.

In a further aspect, the invention is directed to methods for selecting T-cells from a donor which are capable of being transferred into a compatible recipient without causing him a graft-versus-host disease (GVHD) reaction.

In yet another aspect, the invention is directed to methods for treating an hematopoietic cancer wherein CD8+ T-cells from a hematopoietic cancer-free donor are isolated and transferred into a compatible hematopoietic cancerous recipient.

Other objects and advantages of the present invention will be apparent upon reading the following non-restrictive description of several preferred embodiments made with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and **1B** are graphs showing survival of primed (Fig. 1A) and unprimed (Fig. 1B) mice following injection of 10^5 EL4 cells i.v. Priming was performed by i. p. injection of 2×10^7 cells (either normal splenocytes or

EL4 cells) on day –14, or of 300 μg $B6^{dom1}$ peptide (AAPDNRETF; sequence published in Perreault *et al.*, *J. Clin. Invest.*, 98:622, 1996) in incomplete Freund's adjuvant s.c. on day –21 and –14. EL4 cells used for priming were irradiated (10^4 cGy). Ten to fifteen mice per group.

Figure 2 is a graph illustrating the assessment of $B6^{dom1}$-specific $CD8^+$ T-cells by staining with $B6^{dom1}/D^b$ tetramers. Numbers indicate the percentage of $CD8^+$ T-cells that were $tetramer^+$. These data are representative of three to four independent experiments. Staining of splenocytes from unprimed $B10.H7^b$ mice (negative control) as well as $B6^{dom1}$-specific cell line (positive control) shows that $B6^{dom1}/D^b$ tetramers were highly specific and sensitive reagents. The mean percentage (± SD) of $tetramer^+$ $CD8^+$ T-cells was 0.9 ± 0.2 for $B10.H7^b$ mice primed with $B6^{dom1}$ peptide, 2.4 ± 0.4 for $B10.H7^b$ mice primed with B10 cells, and 1.3 ± 0.3 for C3H.SW mice primed with $B6^{dom1}$ peptide. Priming was performed as described in Figure 1.

Figure 3 depicts in graphs that $B6^{dom1}$ is expressed on both hematopoietic and non hematopoietic cells. Peptides were extracted from the liver of B6.PL, $B10.H7^b$, B6.PL→C3H.SW and C3H.SW→B6.PL mice. Extracts containing 5 mg proteins were fractionated by HPLC, and titrated amounts of HPLC fraction 15, in which $B6^{dom1}$ can be found, were incubated with ^{51}Cr-labeled C3H.SW Con A blast targets. Specific cytotoxicity is shown as means of triplicate cultures.

Figure 4 is a graph showing that adoptive transfer of $B6^{dom1}$-primed T-cells can eradicate EL4 cells. Irradiated mice (1000 cGy) were injected i.v. with 10^7 bone marrow cells + 5 x 10^7 splenocytes from $B10.H7^b$ or B10 donors on day 0. Donors were primed with EL4 or B10 cells on day –14 as described in Figure 1. In designation of donor/recipient combinations, the type of cells used for priming is shown in parentheses. Thus, $B10.H7^b$ (B10)→B10 refers to transfer of cells from $B10.H7^b$ donors primed against

B10 cells into irradiated B10 hosts. On day +1, mice received 10^5 EL4 cells i. v. Ten mice per group.

DETAILED DESCRIPTION OF THE INVENTION

A) General overview of the invention

The present invention relates to the elimination of target cells in vertebrates, and more particularly the eradication of hematopoietic cancer cells, by selective transfer of T-cells specific for selected minor histocompatibility antigens (MiHAs). The invention provides T-cells with the potent activity of eliminating target cells when injected into a recipient, without being toxic, *i.e.*, without causing the recipient a graft-versus-host disease (GVHD) reaction. The invention is based on: 1) the priming of T-cells specifically reacting against a selected ubiquitous MiHA, *viz.* a MiHA that is expressed by target cells and by non-target cells of a recipient; and also 2) the selection of a 100% purified population of T-cells that specifically react against a minor histocompatibility antigen which is ubiquitously expressed by the recipient's cells or selectively expressed by specific recipient's target cells only.

MiHA-specific T-cell responses are of remarkable potency and represent the most conclusive documentation that the immune system can cure cancer in human. It was shown recently that anti-MiHAs T-cell responses are so effective that leukemic patients can be cured by AHCT without the need for myeloablative chemo/radiotherapy. Furthermore, many studies of relapsing leukemia patients (with a leukemic burden up to 10^{12} cells) have demonstrated that, without any myeloablative chemotherapy, a single infusion of unprimed MHC matched/MiHA incompatible T-cells is sufficient to obtain a prolonged molecular remission (no leukemic cells detectable by PCR assay) in most patientsNo cancer therapy, of any type, may ever have shown such promise. These results are even more interesting since it is also known that some MiHAs are selectively expressed i) during a specific phase of the cell cycle (*e.g.* the S phase) or during a specific differentiation stage (*e.g.* mature or immature cells from a given lineage), or ii) in viral infected cells.

Thus, selection, priming, and adoptive transfer of T-cells targeted to immunodominant MiHAs could be used to eliminate specifically cancerous cells as well as any abnormal cells and infected cells such as HIV+ infected cells, hepatitis B and hepatitis C infected cells. The present invention encompasses the elimination of all these types of cells.

The present invention encompasses the eradication of target cells in members of the class Vertebrates into which it would be preferable to eliminate such cells. Preferably, the vertebrate is a mammalian subject including, without limitation, human and nonhuman primates, farm animals, domestics animals, laboratory animals.

B) T-cells

In one aspect, the invention is directed to a population of T-cells comprising a plurality of T-cells isolated from a donor and selected for specifically recognizing an immunodominant minor histocompatibility antigen (MiHA) expressed by target cells of a recipient but not expressed by any of the donor's cells. At least some of the T-cells from the T-cells population are capable of being primed against the immunodominant MiHA such that, when transferred into a recipient, the primed T-cells eliminate the recipient's target cells without causing him a graft-versus-host disease (GVHD) reaction.

Preferably, the population of T-cells comprises CD4+ and/or CD8+ T-cells. Indeed, it is well known that not only CD8+, but also CD4+ T-cells can recognize immunodominant MiHAs. More preferably, the T-cells are CD8+ cells. The T-cells may be isolated from a compatible donor using methods well known in the art. Theoretically, the T-cells could also come from a plurality of donors provided that these T-cells recognize specifically the selected immunodominant MiHA(s). According to the invention, selection, priming, and adoptive transfer of such T-cells targeted to immunodominant MiHAs could be very useful to eliminate various types of target cells such as cancerous cells, abnormal cells and virus infected cells. The elimination of these cells could also be the result of a direct effect (a direct attack of the target cells by the T-cells) and/or an indirect effect (via the production of cytokines).

In a more preferred embodiment, the population of T-cells comprises a plurality of CD8+ T-cells isolated from a hematopoietic cancer-free donor and selected for specifically recognizing a ubiquitous and immunodominant minor histocompatibility antigen (MiHA) expressed by hematopoietic cancerous cells of a recipient, but not expressed by any of the donor's cells.

According to the invention, "immunodominant MiHA" refers to an immunogenic amino acid sequence of a selected MiHA (a peptidic portion of the MiHA or the whole protein) that would "prime" the T-cells and cause an effective immune response against the target cells. The MiHAs and fragments thereof useful in the present invention may be purified from living organisms or obtained by synthetic means, *i.e.* chemical synthesis of the polypeptide from component amino acids by methods known to those of ordinary skill in the art. The polypeptide may be obtained by its production in prokaryotic or eukaryotic host cells using techniques known in the art. Immunogenic analogs of MiHAs resulting from amino acid substitution, deletion addition and/or chemical modifications of the MiHA polypeptide are suitable according to the present invention, if these analogs do not materially reduce the immunogenicity of the MiHAs and that they permit to prime efficaciously the donor's T-cells.

The invention encompasses immunodominant MiHAs which are specifically expressed by various cells or tissues and also ubiquitous MiHAs expressed by most or all the recipient's cells. As mentioned hereinbefore, it is known that some MiHAs are specifically expressed by cancerous cells, viral infected cells, and abnormal cell at a specific phase of the cell cycle or at a specific differentiation stage. For instance, some immunodominant MiHAs which are specifically expressed by hematopoietic cells have already been identified. International PCT patent applications published under Nos. WO 97/05169, WO 99/05173 and WO 99/05174 and also U.S. patent No. 5,770,201 disclosed such selectively expressed MiHAs. Similarly, Scott D, *et al,* (*Immunity* 12:711, 2000) recently described the structure of the first MiHA epitope recognized by CD4+ T-cells. These MiHAs as well as others to be discovered yet are suitable according to some aspects of the present invention.

When the selected MiHA is ubiquitous, it is expressed in the recipient not only by its target but also by its non-target cells. The selected MiHA is however not expressed by any of the donor's cells. To avoid a non-specific immune response towards the recipient's non-target cells, it is preferable that the levels of expression for the selected MiHA on non-target cells be no greater than 10% of levels of expression on target cells. More preferably, the ubiquitous MiHA is expressed by the target cells of the recipient at a level of at least about 100 copies per cell. As it will be shown hereinafter in Example 1, an available approach to assess MiHA expression on cells is the titration of peptide extracts in cytotoxic assays. Shirle *et al.* (*Europeen Jour. Immunol.*, 30:2216, 2000) describe another approach that uses mass spectrometry on peptides extracted from tissues or cells suspensions.

Although it has not been tested, it is conceivable that more than one MiHA could be selected and targeted at the same time according to the present invention. Routine experiments could be done by a person skilled in the art in order to determine whether priming of CD8+ T-cells against two or more MiHAs would be better for eliminating target cells or whether it would lead to an immune response, toxic to the recipient. Results would probably depend on the nature of the MiHAs selected and on their levels of expression. Therefore, the present invention encompasses the use of suitable combination(s) of more than one MiHAs.

In a preferred embodiment of the invention, at least some of the T-cells from the population of T-cells have been genetically modified for producing at least one lymphokine. Preferably, the lymphokine is selected from the group consisting of IL-2, IL-4, IL-6, IL-7 and IL-15. More preferably, the T-cells are genetically modified so as to produce IL-15. Methods for genetically modifying eucaryote cells such as T-cells are well known in the art.

C) Therapeutic physiological solutions

In another aspect, the invention is directed to a therapeutic physiological solution for eliminating target cells of a recipient, and comprising: a population of T-cells as defined hereinbefore, and a physiologically acceptable diluent. Preferably, the therapeutic physiological solution is used for eliminating

hematopoietic cancerous cells of a hematopoietic cancerous recipient. Such anti-cancerous solution comprises a physiologically acceptable diluent and CD8+ T-cells isolated from a compatible hematopoietic cancer-free donor as defined hereinbefore. Physiologically acceptable diluents include saline and aqueous buffer solutions.

The therapeutic physiological solution may further comprise therapeutically active agents such as anti-inflammatory agents, compounds modulating immunity, growth factors, nucleic acids (DNA, anti-sense, RNA), antibodies, etc. It may also contain preserving agents, solubilizing agents, stabilizing agents, wetting agents, emulsifiers, colorants, salts, buffers, coating agents or antioxidants. The therapeutic physiological solution of the invention may be administered by any suitable route and the amount to be administered is that amount necessary for inducing an effective immune response towards the selected MiHA and eliminating the target cells of the recipient. Suitable dosages will vary, depending upon factors such as the type and amount of T-cells in the solution, the type of disease to be treated, the nature of the selected MiHA(s), the route of administration and the age and weight of the individual to be treated. Dosage regimens could be adjusted to provide the optimum therapeutic response. The therapeutic physiological solution of the invention would be prepared using methods well known in the art, preferably in the form of a sterile injectable aqueous solution.

D) Methods for selecting T-cells

In a further aspect, the invention is directed to a method for selecting T-cells from a donor which are capable of being transferred into a compatible recipient. This method is very useful since the selected T-cells are capable of being transferred into the recipient without causing a graft-versus-host-disease (GVHD) reaction. More particularly the method comprises the steps of:

a) selecting an immunodominant minor histocompatibility antigen (MiHA) expressed by target cells of the recipient but not expressed by any of the donor's cells, wherein the immunodominant MiHA is expressed by the recipient's target cells at a level of at least about 100 copies per cell;

b) isolating T-cells from the donor; and

c) • positively selecting, from the T-cells isolated at step b), T-cells that specifically recognize the immunodominant MiHA of step a); and/or

• eliminating, from the T-cells isolated at step b), T-cells reacting against any immunodominant minor histocompatibility antigen(s) other than the immunodominant MiHA of step a);

whereby the T-cells selected/not eliminated at step c) are capable of being transferred into a compatible recipient without causing him a graft-versus-host disease (GVHD) reaction. More preferably, step c) is performed so that 100% of the T-cells selected/not eliminated specifically recognize said immunodominant MiHA.

In a preferred embodiment, the T-cells are selected from CD4+ and CD8+ T-cells and the recipient's target cells are selected from the group consisting of cancerous cells, viral infected cells, and abnormal cells at a specific phase of the cell cycle or at a specific differentiation stage.

According to another preferred embodiment, the selection method is used for selecting CD8+ T-cells from a hematopoietic cancer-free donor which are capable of eliminating hematopoietic cancerous cells when transferred into a compatible hematopoietic cancerous recipient without causing him a graft-versus-host disease (GVHD) reaction. This preferred method comprises the steps of:

a) selecting a ubiquitous and immunodominant minor histocompatibility antigen (MiHA) expressed by hematopoietic cells of the recipient but not expressed by any of the cells of the donor;

b) isolating CD8+ T-cells from the donor;

c) • positively selecting, from the T-cells isolated at step b), the CD8+ T-cells that specifically recognize the MiHA of step a); and/or

• eliminating, from the T-cells isolated at step b), the CD8+ T-cells reacting against immunodominant minor histocompatibility antigen(s) other than the MiHA of step a); and

d) priming *in vitro* the CD8+ T-cells selected/not eliminated at step c) against the MiHA of step a);

whereby, the CD8+ T-cells primed at step d) are capable of being transferred into a compatible hematopoietic cancerous recipient for eliminating hematopoietic

cancerous cells of said recipient without causing him a graft-versus-host disease (GVHD) reaction.

The selection method may further comprise the step of priming *in vitro* the T-cells selected/not eliminated at step c) against the selected MiHA such that, when transferred into the recipient, the primed T-cells eliminate the target cells of the recipient.

The selection method may also further comprise the step of genetically modifying the T-cells isolated from said donor such that they produce at least one lymphokine selected from the group consisting of IL-2, IL-4, IL-6, IL-7 and IL-15.

E) Methods of treatment

The invention is directed to an improved method for eliminating, in a recipient, target cells expressing a selected immunodominant minor histocompatibility antigen (MiHA), without causing the recipient a graft-versus-host-disease reaction; the known method comprising the steps of:

- isolating T-cells from a compatible donor;
- transferring the T-cells to the recipient; and
- priming the donor's T-cells against the immunodominant minor histocompatibility antigen (MiHA);

the improvement wherein at least one of the following conditions is satisfied:

- 100% of the T-cells transferred from said donor into said recipient specifically recognize said immunodominant MiHA;
- said immunodominant MiHA is expressed by all the cells of the recipient but is not expressed by any of the cells of said donor.

In one of its most practical aspect, the invention is directed to an improved method for treating an hematopoietic cancer wherein CD8+ T-cells from a hematopoietic cancer-free donor are isolated and transferred into a compatible hematopoietic cancerous recipient. The known method is improved in that the CD8+ T-cells from the donor are primed against a ubiquitous minor histocompatibility antigen (MiHA) expressed by all the recipient's cells (including hematopoietic cancerous cells), but not expressed by any of the donor's cells.

In another very practical aspect, the invention is directed to a method for treating an hematopoietic cancer comprising the steps of:

- proceeding to a hematopoietic stem-cells transplant from a hematopoietic cancer-free donor to a compatible hematopoietic cancerous recipient;
- selecting a ubiquitous and immunodominant minor histocompatibility antigen (MiHA) expressed by hematopoietic cancerous cells of the recipient but not expressed by hematopoietic cells of the donor;
- isolating CD8+ T-cells from the donor and injecting these isolated CD8+ T-cells into the recipient; and
- priming the donor's CD8+ T-cells against the selected ubiquitous immunodominant MiHA such that these primed CD8+ T-cells eliminate the hematopoietic cancerous cells of the recipient.

In yet another aspect, the invention is directed to an improved method for treating an hematopoietic cancer comprising the steps of:

- isolating CD8+ T-cells from a hematopoietic cancer-free donor;
- transferring the isolated CD8+ T-cells to a hematopoietic cancerous recipient; and
- priming the donor's CD8+ T-cells against a minor histocompatibility antigen (MiHA) selectively expressed by hematopoietic cells;

the improvement wherein 100% of the CD8+ T-cells transferred from the donor into the recipient specifically recognize the MiHA selectively expressed by the hematopoietic cells.

In still another practical aspect, the invention is directed to a method for treating an hematopoietic cancer comprising the steps of:

- proceeding to a hematopoietic stem-cells transplant from a hematopoietic cancer-free donor to a compatible hematopoietic cancerous recipient;
- selecting a minor histocompatibility antigen (MiHA) selectively expressed by hematopoietic cells;
- isolating CD8+ T-cells from the donor;
- injecting into the recipient a population of isolated CD8+ T-cells wherein 100% of the CD8+ T-cells specifically recognize the selected MiHA; and

- priming the donor's CD8+ T-cells against the selected MiHA such that these primed lymphocytes eliminate the hematopoietic cancerous cells of the recipient.

The CD8+ T-cells are generally transferred following a hematopoietic stem-cells transplantation from an hematopoietic cancer-free donor to a compatible hematopoietic cancerous recipient. Such transplantation is performed according to regular methods well known in the art of hematopoietic stem-cells purification and transplantation. For instance, it is well known in human tissue transplantation, that compatibility between a donor and a recipient depends on the number of HLA genes they shared, and that in order to be compatible, the donor and the recipient must have at least some HLA genes in common. According to the present invention, it is preferable, that the donor and the recipient be HLA genotypically identical.

The CD8+ T-cells to be used are isolated from the donor and injected into the recipient using well known methods. Theoretically, the T-cells could, if necessary, be isolated from more than one compatible donor as explained previously. The CD8+ T-cells are also preferably "primed" against a selected minor histocompatibility antigen (MiHA) in order to elicit an immune response towards the selected MiHA and lead to the elimination of the hematopoietic cancerous cells expressing the selected MiHA. Priming of CD8+ T-cells is performed by exposing the CD8+ T-cells of the donor to an immunogenic amino acid sequence of the selected MiHA (a peptidic portion of the MiHA or the whole protein) using methods within the skill of the art. For practical and efficacy reasons, it is preferable that the priming be performed *in vitro* prior to transfering the CD8+ T-cells into the recipient. However, it is also possible to prime the lymphocytes *in vivo* subsequently to their transfer into the recipient or before their isolation from the donor. *In vivo* priming can be done by injecting into the donor/recipient the whole MiHA or a peptidic portion thereof, preferably in combination with an adjuvant.

According to the invention, it is also highly preferable that 100% of the T-cells transferred from the donor into the recipient specifically recognize the selected MiHA (ubiquitous or specific to the target cells). Indeed, Example 1 hereinbelow demonstrates that, for reducing to a minimum any risk of GVHD, one

must take specific measures to deplete T-cells reactive to recipient antigens other than the target MiHA in all donor-derived hematopoietic cell preparations injected to the recipient before or at the time of immunotherapy. Therefore, in a preferred embodiment of the invention, all T-cells reacting against minor histocompatibility antigen(s) other than the selected MiHA are eliminated prior to their transfer into the recipient. To achieve such selection/elimination, the methods of the invention preferably further comprise at least one step selected from the steps of:

a) positively selecting isolated CD8+ T-cells that specifically recognize the selected MiHA and transferring solely into the recipient these positively selected CD8+ T-cells; and

b) eliminating CD8+ T-cells reacting against immunodominant minor histocompatibility antigen(s) other than the recipient's selected MiHA prior to transferring the donor's CD8+ T-cells into the recipient.

The selection/elimination steps can be done using methods well known in the art such as flow cytometry and *in vitro* expansion of CD8+ T-cells specifically recognizing the selected MiHA.

The CD8+ T-cells selected/not eliminated are preferably injected to a recipient in need of treatment for hematopoietic cancers and therefore, the steps of selecting the MiHA, selecting/eliminating the CD8+ T-cells and genetically modifying the CD8+ T-cells can be done as described hereinbefore in the aspect concerning the population T-cells.

In a preferred embodiment of the invention, the methods for treating hematopoietic cancers further comprise the step of preventing activation induced cell death (AICD) of the CD8+ T-cells transferred into the recipient. A way to achieve this result, is to genetically modify at least some of the CD8+ T-cells prior to their transfer such that they produce at least one lymphokine.

Although methods for treating hematopoietic cancers have been described in detail hereinbefore, the present invention encompasses other adoptive immunotherapy methods for eliminating other types of cells. Indeed, it is believed that a person skilled in the art could modify and adapt the present anti-cancerous adoptive immunotherapy methods for treating other types of diseases without undue experimentation.

B) EXAMPLE 1: Eradication of hematopoietic cancer cells in mice using $B6^{dom1}$ MiHA

As it will now be demonstrated by way of an example hereinafter, the invention provides T-cells specifically recognizing minor histocompatibility antigen(s), methods for selecting these cells and uses thereof for eliminating target cells and more particularly to eliminate hematopoietic cancers cells.

The following example is illustrative of the wide range of applicability of the present invention and is not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred methods and materials are described.

1) Background

The Applicant has previously described the mouse $B6^{dom1}$ MiHA, an $H2D^b$-associated nonapeptide encoded by the *H7* locus at the telomeric end of chromosome 9. $B6^{dom1}$ ($H7^a$) is expressed at extremely high levels (1,000 copies/cell) on hematopoietic cells. Initially, based on previously published work, it was not believed that $B6^{dom1}$ represented a good target for immunotherapy of hematopoietic malignancies since this MiHA peptide was present in practically all tissues and organs. Considering the wide tissue distribution of $B6^{dom1}$, the Applicant was expecting that transfer of $B6^{dom1}$-reactive T-cells would cause severe GVHD. This assumption was strengthened when it was observed that several mice injected with T-cells from C3H.SW donors primed with $B6^{dom1}$ peptide preparations presented mild signs of cutaneous GVHD.

2) Materials and methods

Mice. The following strains of mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in the Guy-Bernier Research Center: B6.PL-*Thy-1*^{a}/Cy (B6.PL), C3H/HeJ, C3H.SW, C57BL/6J (B6), C57BL/10J (B10), B10.C-*H7*^{b}(47N)/Sn ($B10.H7^b$) and LP/J. Mice were used between 6 and 16 weeks of age

and were maintained in specific pathogen-free conditions according to the standards of the Canadian Committee for Animal Protection.

Tumor cells and CD8$^+$ T-cell lines. EL4 thymoma cell line (of C57BL/6J origin) was obtained from the American Type Culture Collection (Rockville, MD). The B6^{dom1}-specific T-cell lines have been previously described (Eden PA *et al. J. Immunol.* 162:4502, 1999).

Cell transplantation and assessment of GVHD. Mice were transplanted as described previously (Fontaine P *et al, Immunogenetics* 34:222, 1991). In most experiments, recipient mice received 1000 cGy total body irradiation from a ^{60}Co source at a dose rate of 128 cGy/min on day 0, the day of transplant. The dose of irradiation was increased to 1200 cGy for production of chimeras used in experiments depicted in Figure 3. Bone marrow cells were obtained from the tibiae and femurs of donor mice. Bone marrow cells (10^7) mixed with spleen cells (5 x 10^7) were given as a single intravenous injection, via the tail vein, in a volume of 0.5 ml of serum-free RPMI-1640 medium. Transplanted mice were observed twice a week for skin signs of GVHD (hair loss, dermatitis), and the day of death was recorded.

Synthetic peptides. B6^{dom1} peptide (AAPDNRETF; sequence published in Perreault *et al.*, *J. Clin. Invest.*, 98:622, 1996) was synthesized by the Sheldon Biotechnology Center (Montreal, Canada). Purity, as determined by reversed phase-high performance liquid chromatography and mass spectrometric analysis, was above 97%.

Extraction and HPLC fractionation of natural B6^{dom1} peptide. B6^{dom1} peptide was extracted from tissue homogenates in 0.1% trifluoroacetic acid, in the presence of protease inhibitors (25 mM iodoacetamide, 1mM aprotinine, 1mM PMSF) as described previously (Eden PA *et al. J. Immunol.* 162:4502, 1999). After a pre-purification on a C18 SEP-PAK™ column (Waters, Milford, MA) extracts containing 5 mg proteins were fractionated on an HPLC system using a LUNA™ C18 column

(5 μm, 4.6 x 250 mm, Torrance, CA). Solvents used were 99.9% water/0.1% trifluoro-acetic acid (solvent A) and 99.9% acetonitrile/0.1% trifluoro-acetic acid (solvent B). The gradient consisted of the following linear step intervals: 0% B (0-5 minutes), up to 20% B at 10 minutes, up to 55% B at 55 minutes, plateau at 55% B from 55 to 60 minutes, and up to 100% B at 70 minutes. Flow rate was 1 ml/minute, and 1 ml fractions were collected and lyophilized. To evaluate the presence of $B6^{dom1}$ in tissue extracts, fraction 15, where the $B6^{dom1}$ peptide eluted, was incubated with ^{51}Cr-labeled C3H.SW Con A blast targets which were tested in cytotoxicity assays in the presence of $B6^{dom1}$-specific effectors (effector/target cell ratio was 10/1). Lysis of target cells was measured as specific cytolysis, based on the level of ^{51}Cr released into the supernatant relative to spontaneous and maximal ^{51}Cr release (Fontaine P *et al*, *Immunogenetics* 34:222, 1991).

MHC class I/peptide tetramers. MHC class I ($H2D^b$)/peptide ($B6^{dom1}$) tetramers were produced as previously described (Altman JD *et al.*, *Science* 274:94, 1996; and Gallimore A *et al.*, *J. Exp. Med.* 187:1383, 1998). Recombinant β_2-microglobulin and the extracellular domain of the MHC class I heavy chain containing the BirA recognition sequence in frame at its C terminus were overexpressed in *Escherichia coli* as insoluble aggregates that formed inclusion bodies. Purified inclusion bodies were solubilized in urea, and monomeric $H2D^b$ complexes were refolded around peptide by dilution of denaturing buffer. Monomeric complexes were recovered by anion exchange chromatography over a MONO Q HR™ column (Pharmacia, Upsala, Sweden). $H2D^b$/peptide complexes were biotinylated using BirA enzyme (Avidity, Denver, CO) as described. Tetramers were generated by mixing the biotinylated monomeric complexes with NEUTRAVIDIN-PE™ (Molecular Probes, Eugene, OR) at a 6:1 molar ratio. Then, biotinylated tetramers were purified by gel filtration over a SUPERDEX 200 HR™ column (Pharmacia). Purified tetramers were stored at 1 mg/ml at 4°C and were frequently tested on the $B6^{dom1}$-specific T-cell line to document maintenance of staining capacity and signal intensity.

Cell staining and flow cytometry. Evaluation of chimerism was performed with PE-conjugated anti-Thy-1.2 (clone 30-H12) and FITC-conjugated anti-Thy-1.1 (clone OX-7) antibodies from Pharmingen (Mississauga, Canada). For estimation of tetramer$^+$ splenocytes, cell suspensions (100µl) were stained with anti-CD8 (APC) antibody (clone 53-6.7; Pharmingen) in PBS/BSA 0.1% for 25 minutes at 4°C, washed, and then incubated with 1µg PE-labeled tetramers at 37°C for 40 minutes. TCR expression was monitored for antigen specific T-cell lines with anti-TCRαβ (FITC) antibody (clone H57-597; Pharmingen). Cells were analyzed on a FACSCALIBUR™ (Becton Dickinson, Mountain View, CA) using CELLQUEST™ software.

3) Results

Priming against B6^{dom1} (H7^a) confers resistance to EL4 thymoma cells. The Applicant first addressed the question as to whether an immune response targeted to the immunodominant B6^{dom1}/H7^a MiHA would confer resistance to (B6-derived) EL4 leukemic cells. It is known that B6^{dom1} is absent on cells from B10.H7^b, C3H/HeJ and C3H.SW mice, but is present on B6, B10, EL4 and LP cells. It is also known that EL4 cells do not express MHC class II antigens, but express MHC class I antigens as well as six tumor specific MHC class I-associated CTL epitopes. As shown in Figure 1A, B6 mice injected with 10^5 EL4 cells died within 40 days. Thus, TAAs found on EL4 cells do not elicit protective immune response in unprimed animals.

B10 mice differ from B6 mice (and EL4 cells) at the *H9* locus which encodes a non-dominant MiHA; B10 have the *H9*^{a} allele while B6 have a "non a" allele (*H9*$^{-a}$). As shown in Figure 1A, host vs EL4 disparity at the *H9* locus had no effect since survival of B10 recipients was not prolonged relative to that of B6 hosts. In contrast, survival of recipients with disparity either for the *H7* locus (which encodes the immunodominant B6^{dom1} MiHA) or the full MHC (C3H/HeJ) was 30% and 100%, respectively.

Figure 1B shows results wherein the susceptibility to EL4 cells in mice primed against specific antigens was assessed. Resistance to EL4 cells and survival increased slightly, to 20%, in mice primed against H9^{-a} + EL4 TAAs.

Strikingly, survival reached 70% and 100% in mice primed with synthetic $B6^{dom1}$ peptide and $B6^{dom1}$-positive cells, respectively. In the latter group, resistance to EL4 cells was significantly enhanced as compared to unprimed animals ($p = 0.003$, Fisher's exact test). Besides, injection of $B6^{dom1}$-positive cells seemed slightly more effective than peptide immunization in conferring resistance to EL4 cells.

Together, these results indicate that an immuno-dominant MiHA ($B6^{dom1}$) can trigger a very potent immune response which is more protective than that elicited by TAAs and a non-dominant MiHA (H9), and whose efficacy is increased by prior antigen priming.

Selective transfer of anti-$B6^{dom1}$ T-cells does not cause GVHD. Having shown that $B6^{dom1}$ elicits protective anti-cancer immune response, the question arises as to whether transfer of $B6^{dom1}$-specific T-cells would cause GVHD. Thus, the Applicant transferred 10^7 bone marrow cells (as a source of hematopoietic progenitors) and 5×10^7 splenocytes (as a source of T-cells) from either primed (against $B6^{dom1}$) or unprimed donors, and assessed survival and skin signs of GVHD in irradiated B10 recipients. Two types of donors were used: $B10.H7^b$ mice which differ from B10 hosts at the H7 locus, and C3H.SW mice which differ from B10 for numerous MiHAs including H7.

Strikingly, transfer of $B10.H7^b$-derived cells from either naïve or primed donors caused no GVHD in B10 hosts (see Table I hereinafter). Lack of GVHD was observed with cells harvested from $B10.H7^b$ donors primed under three conditions: s.c. injection of $B6^{dom1}$ peptide in adjuvant, or i.p. injection of splenocytes presenting selective $B6^{dom1}$ difference (B10 cells) or multiple MiHA differences including $B6^{dom1}$ (LP cells).

TABLE I: Evaluation of GVHD induced by cells from naïve or $B6^{dom1}$-primed donors

Donor[1]	Priming[2]	Recipient[3]	Signs of GVHD Dermatitis (%)	Day 100 survival (%)
$B10.H7^b$	none	B10	0	100
$B10.H7^b$	$B6^{dom1}$ peptide	B10	0	100
$B10.H7^b$	B10 cells	B10	0	100
$B10.H7^b$	LP cells	B10	0	100
C3H.SW	none	B10	0	100
C3H.SW	$B6^{dom1}$ peptide	B10	53	80
C3H.SW	$B6^{dom1}$ peptide	$B10.H7^b$	0	100

[1] 10^7 bone marrow cells + 5 x 10^7 spleen cells.

[2] 300 µg $B6^{dom1}$ peptide (AAPDNRETF) in incomplete Freund's adjuvant s.c. day –21 and –14, or 2 x 10^7 splenocytes i. p. on day –14.

[3] Total body irradiation (10 Gy) on day 0. 15 mice per group.

The inability of $B10.H7^b$-derived anti-$B6^{dom1}$ T-cells to elicit GVHD was seemingly at odds with the previous published observation that mild signs of GVHD can be observed in a significant proportion of B6 mice following injection of cells from C3H.SW donors primed by s.c. injection of $B6^{dom1}$ peptide in adjuvant. Thus, a series of experiments was performed to understand why GVHD was observed following transplantation of $B6^{dom1}$-primed cells from C3H.SW but not from $B10.H7^b$ donors (see Table I). In B10 hosts, the occurrence of mild GVHD following injection of cells from C3H.SW mice primed with $B6^{dom1}$ peptide, but not from unprimed donors was observed. Notably (see Table 1), cells from C3H.SW donors primed with $B6^{dom1}$ peptide elicited no GVHD in $B10.H7^b$ recipients. This result shows that expression of $B6^{dom1}$ by host cells is absolutely necessary for induction of GVHD by $B6^{dom1}$-primed C3H.SW-derived T-cells. This rules out the possibility of GVHD being initiated by T-cells reactive to some contaminant or cross-reactive peptide in the $B6^{dom1}$ peptide preparation.

An alternative hypothesis would be that following antigen priming, expansion of $B6^{dom1}$-reactive T-cells is more important in C3H.SW than in $B10.H7^{b}$ mice. Results presented in Fig. 2 exclude the latter premise: Figure 2 shows that the percentage of $B6^{dom1}$-specific T-cells was not greater in peptide-primed C3H.SW donors than in $B10.H7^{b}$ donors primed with B10 cells 14 days after priming. Besides, comparison of $B10.H7^{b}$ mice primed with $B6^{dom1}$-positive cells or with $B6^{dom1}$ peptide showed that, as suggested by results depicted in Figure 1, expansion of $tetramer^{+}$ T-cells was greater after cell injection than after peptide immunization.

Results of the above experiments left a single important difference between C3H.SW- and $B10.H7^{b}$-derived T-cells: only the former contain T-cell precursors reactive to MiHAs other than $B6^{dom1}$ on B10 cells. Thus, a logical inference is that the occurrence of a mild form of GVHD in B10 recipients of T-cells from $B6^{dom1}$-primed C3H.SW donors is best explained by epitope spreading, a known process whereby epitopes distinct from and non-cross-reactive with an inducing epitope become targets of an evolving immune response. According to this model, destruction of host hematopoietic cells by $B6^{dom1}$-primed T-cells has no deleterious consequence *per se* (c.f. $B10.H7^{b}$ donors). However, the ensuing inflammatory reaction provides an ideal milieu to prime T-cells specific for other host MiHAs (provided they are present in the injected inoculum, c.f. C3H.SW donors), and initiate GVHD.

$B6^{dom1}$ MiHA is expressed ubiquitously on both hematopoietic and non-hematopoietic cells. Previous studies in mouse hematopoietic chimeras suggest that, in order to trigger GVHD, MiHAs must be expressed on both hematopoietic and non-hematopoietic host cells. In keeping with this idea, could the inability of $B6^{dom1}$ to elicit GVHD be explained by selective expression on hematopoietic cells? By testing peptide extracts from a variety of B6 organs, previous studies have shown that $B6^{dom1}$ has a wide tissue distribution since it was recovered in all organs tested. However, this does not prove that $B6^{dom1}$ is present on non-hematopoietic cells. An alternative explanation would be that $B6^{dom1}$ recovered from non lymphoid organs derives from resident hematopoietic cells (lymphocytes,

macrophages, dendritic cells). To discriminate between these two possibilities, the Applicant created hematopoietic chimeras that expressed $B6^{dom1}$ either on hematopoietic cells or non-hematopoietic cells. Thus, irradiated (1200 cGy) C3H.SW (Thy1.2, $B6^{dom1}$ neg) mice were transplanted with B6.PL (Thy1.1, $B6^{dom1}$ pos) bone marrow + spleen cells (B6.PL→C3H.SW), and vice versa (C3H.SW→B6.PL). When tested, on day 180 post-transplant, mice were complete hematopoietic chimeras as determined by flow cytometry analysis of spleen cells with anti-Thy1.1 and anti–Thy1.2 antibodies (< 1% host cells; data not shown). When titrated amounts of peptide extracts where used to sensitize target cells to lysis by $B6^{dom1}$-specific T-cells, $B6^{dom1}$ was recovered from the liver of B6 but not from $B10.H7^{b}$ mice. This indicates that the $B6^{dom1}$-specific T-cell line does not crossreact with other peptides from the C57BL background (Figure 3). Notably, $B6^{dom1}$ was present in similar amounts in liver extracts from B6.PL→C3H.SW and C3H.SW→B6.PL mice. Hence, $B6^{dom1}$ is present in both hematopoietic and non-hematopoietic cells. Similar results were obtained when peptides were extracted from the lung (data not shown). Titration of peptide extracts in cytotoxic assays, the standard available approach to assess MiHA expression, provides only gross semiquantitative estimates. Nevertheless, considering that i) similar amounts of $B6^{dom1}$ were extracted from non-hematopoietic cells (C3H.SW→B6.PL liver) relative to hematopoietic cells (B6.PL→C3H.SW liver), and that ii) the number of hematopoietic cells, essentially Küpffer cells, is less than 10% than that of non-hematopoietic cells (hepatocytes + endothelial cells), it can be estimated that, on a per cell basis, the mean number of $B6^{dom1}$ copies on liver non hematopoietic cells is no more than one tenth than that on hematopoietic cells.

Eradication of leukemic cells following adoptive transfer of $B6^{dom1}$-reactive T-cells.

Adoptively transferred anti-$B6^{dom1}$ T-cells do not elicit GVHD, but can they eliminate neoplastic cells? Irradiated mice (1000 cGy) were injected i. v. with 10^7 bone marrow cells + 5 x 10^7 splenocytes from $B10.H7^{b}$ or B10 donors on day 0, and 10^5 EL4 cells on day +1. Donors had been primed against $B6^{dom1}$ by i. p. injection of B10 spleen cells on day –14. Recipients were either $B10.H7^{b}$ or B10 mice; in the former group, $B6^{dom1}$ was present only on injected EL4 cells, whereas

in the latter, it was also present on host cells. Mice were observed for skin signs of GVHD, and autopsy was performed on mice dying before day 100 to ascertain whether their demise was due to GVHD (lymphoid atrophy) or leukemia (hepatosplenomegaly and enlarged lymph nodes).

Figure 4 shows that, as expected, mice that received T-cells from B10 donors (i. e., containing no anti-$B6^{dom1}$ T-cells) were not protected and died of EL4 leukemia between day 23 and 43. In contrast, long-term survival was achieved in 60% of B10 recipients injected with $B6^{dom1}$-primed $B10.H7^{b}$-derived T-cells; no GVHD was observed, but 40 % of recipients died of leukemia. Notably, the cure rate was increased to 100% in $B10.H7^{b}$ recipients injected with the same inoculum; this difference between B10 and $B10.H7^{b}$ hosts was significant ($p = 0.03$, Fisher's exact test). By comparison, transfer of T-cells from B10 donors primed against EL4 TAAs yielded only 20% long-term survival (8 out of 10 recipients died of leukemia), significantly less than the survival rate of $B10.H7^{b}$ recipients of $B6^{dom1}$-primed T-cells ($P = 0.0007$, Fisher's exact test). Thus, while adoptive transfer of anti-$B6^{dom1}$ T-cells did not cause GVHD, it produced a potent GVL response, more effective than that achieved by transferring T-cells primed against TAAs. Based on staining with $B6^{dom1}/D^{b}$ tetramers (Figure 2), it was calculated that the number of $B6^{dom1}$-specific $CD8^{+}$ T-cells in the grafted inoculum was about 2×10^{5}. Hence, eradication of leukemia cells was obtained under conditions where the effector ($B6^{dom1}$-specific T-cells) to target (EL4 cells) ratio was approximately 2:1. The difference in survival between B10 and $B10.H7^{b}$ recipients of $B6^{dom1}$-primed T-cells indicates that tissue distribution of the target MiHA influences the potency of the GVL reaction.

4) Conclusion

This work provides, for the first time, the proof of principle that successful MiHA-specific adoptive immunotherapy of hematological malignancies can be achieved without GVHD. Furthermore, the observations herein raise several unforeseen issues regarding the avoidance of GVHD and the potentiation of GVL, respectively. The Applicant showed that in order to obtain optimal results, MiHA

based immunotherapy requires i) selection and manipulation of specific effector T-cells and ii) targeting of MiHAs with a specific expression profile.

Several important points can be made from the data presented herein:

A) Targeting of a single MiHA is sufficient to eradicate leukemia cells.

B) Not all MiHAs are adequate targets for immunotherapy. Targeting a non dominant MiHA (H9) had little effect. Only an immunodominant MiHA ($B6^{dom1}$) elicited an immune response powerful enough to eradicate leukemia cells. In previous studies, it has been shown that the immunodominant status of $B6^{dom1}$ is due to its high expression level on hematopoietic cells (1000 copies/cell). When its expression level is decreased tenfold, $B6^{dom1}$ looses its immunodominant status. Thus, it is proposed that MiHA based immunotherapy should be targeted at MiHAs expressed at $\geq$ 100 copies per cell.

C) The literature is entrenched with the view that avoidance of GVHD will require targeting MiHAs present strictly on hematopoietic cells. This represents a significant obstacle since it is quite complicated to uncover immunodominant MiHAs that are strictly expressed on hematopoietic cells. Therefore, finding that anti-$B6^{dom1}$ T-cells do not cause GVHD, even though this MiHA has a wide tissue distribution, is particularly good news and was totally unexpected. This observation shows that, compared with hematopoietic cells, non-hematopoietic cells are less susceptible to MiHA-specific T-cells. Differential susceptibility to MiHA-reactive T-cells is presumably related to the fact that the amount of MiHA peptide on the cell surface is determined by the level of expression of MHC molecules which is lower on non-hematopoietic than hematopoietic cells. This idea is consistent with Applicant's estimate of $B6^{dom1}$ expression being one order of magnitude lower on hepatocytes relative to Küpffer cells. Accordingly, the Applicant infers that presence of an immunodominant MiHA on non-hematopoietic cells does not preclude its selection as an immunotherapy target provided its expression level is not above 10% than that of hematopoietic cells.

D) With respect to GVHD induction by $B6^{dom1}$-primed donors, no GVHD was observed when $B6^{dom1}$-specific T-cells were the only host-reactive T-cells present in the grafted inoculum ($B10.H7^{b}$ donors). However, a mild form of GVHD, most

consistent with epitope spreading, was detected when naïve T-cells reactive to other host MiHAs (which did not cause GVHD when transplanted alone, cf. naïve C3H.SW donors) were co-injected with $B6^{dom1}$ specific T-cells. The most plausible explanation is that destruction of host hematopoietic cells by $B6^{dom1}$-primed T-cells has no deleterious consequence *per se*, but promotes cross presentation of host MiHAs in an inflammatory context (the known posttransplant "cytokine storm"). Accordingly, this cross presentation of host MiHAs would have no consequence when only anti-$B6^{dom1}$ T-cells are transferred, but would result in the priming of co-injected T-cells specific for other MiHAs, and thereby initiate GVHD. This leads the Applicant to infer that avoidance of GVHD will require that specific measures be taken to deplete T-cells reactive to host antigens other than the target MiHA in all donor-derived hematopoietic cell preparations injected to the recipient before or at the time of immunotherapy. Enrichment of T-cells reactive to the target MiHA and elimination of other host reactive T-cells could be achieved by methods such as flow cytometry sorting of T-cells labeled with HLA/MiHA dimers or tetramers, and/or by *in vitro* expansion of MiHA-reactive T-cells following stimulation with the target MiHA presented on cells or artificial surfaces.

E) Following priming against $B6^{dom1}$ (on B10 splenocytes), $B10.H7^{b}$ mice showed 100% resistance to a lethal inoculum of EL4 cells (Fig. 1), and eradicated leukemic cells following adoptive transfer in 100% of syngeneic hosts (Fig. 3). However, the cure rate was decreased to 60% when these T-cells were injected into B10 hosts *i.e.,* when the target MiHA was expressed not only on leukemic cells but also on host cells. This shows that the fate of adoptively transferred T-cells, and thus the efficacy of the GVL effect, is influenced by the target antigen load encountered in the recipient. It has been shown in numerous models that high antigen load causes AICD of antigen-reactive T-cells. In the case of adoptively transferred T-cells reactive to host histocompatibility antigens, apoptosis of alloreactive T-cells is massive, rapid, and caused mainly by Fas/FasL interactions. Accordingly, since many MiHAs have a wide tissue distribution, it is deduced that preventing AICD of adoptively transferred T-cells will represent an important adjunct to successful MiHA-based leukemia immunotherapy. It has been shown that AICD of activated/memory T-cells can be inhibited by cytokines of the IL-2

family (IL-2, IL-4, IL-7, and IL-15) and by IL-6. The Applicant therefore proposes that *in vitro* transfection into donor-derived T-cells of cDNA coding for cytokines of the IL-2 family and/or for IL-6 should be a most appropriate strategy to enhance the survival and GVL efficacy of adoptively transferred T-cells targeted to MiHA with a wide tissue distribution.

While several embodiments of the invention have been described, it will be understood that the present invention is capable of further modifications, and this application is intended to cover any variations, uses, or adaptations of the invention, following in general the principles of the invention and including such departures from the present disclosure as to come within knowledge or customary practice in the art to which the invention pertains, and as may be applied to the essential features hereinbefore set forth and falling within the scope of the invention or the limits of the appended claims.

CLAIMS:

1. A population of T-cells comprising a plurality of T-cells isolated from a donor and selected for specifically recognizing an immunodominant minor histocompatibility antigen (MiHA) expressed by target cells of a recipient but not expressed by any of the donor's cells, at least some of the T-cells from said population being capable of being primed against said immunodominant MiHA such that, when transferred into a recipient, the primed T-cells eliminate the recipient's target cells without causing the recipient a graft-versus-host disease (GVHD) reaction.

2. The population of T-cells according to claim 1, wherein said immunodominant MiHA is ubiquitous.

3. The population of T-cells according to claim 2, wherein levels of expression of said ubiquitous MiHA by said target cells are substantially higher than levels of expression for said MiHA by non-target cells of the recipient.

4. The population of T-cells according to any one of claims 1 to 3, wherein said immunodominant MiHA is expressed by the recipient's target cells at a level of at least about 100 copies per cell.

5. The population of T-cells according to any one of claims 1 to 4, wherein at least some of the T-cells from said population have been genetically modified for producing at least one lymphokine.

6. The population of T-cells according to claim 5, wherein said lymphokine is selected from the group consisting of IL-2, IL-4, IL-6, IL-7 and IL-15.

7. The population of T-cells according to any one of claims 1 to 6, wherein said T-cells are CD4+ or CD8+ T-cells.

8. The population of T-cells according to any one of claims 1 to 7, wherein the recipient's target cells are selected from the group consisting of cancerous cells, viral infected cells, and abnormal cells at a specific phase of the cell cycle or at a specific differentiation stage.

9. The population of T-cells according to any one of claims 1 to 8, wherein the donor and the recipient are humans.

10. A therapeutic physiological solution for eliminating target cells of a recipient, comprising: a population of T-cells according to any one of claims 1 to 9, and a physiologically acceptable diluent.

11. A population of T-cells comprising a plurality of CD8+ T-cells isolated from a hematopoietic cancer-free human donor and selected for specifically recognizing a ubiquitous and immunodominant minor histocompatibility antigen (MiHA) expressed by hematopoietic cancerous cells of a human recipient, but not expressed by any of the donor's cells.

12. A therapeutic physiological solution for eliminating hematopoietic cancerous cells of a hematopoietic cancerous human recipient, comprising: a population of T-cells according to claim 11, and a physiologically acceptable diluent.

13. A method for selecting T-cells from a donor which are capable of being transferred into a compatible recipient without causing the recipient a graft-versus-host disease (GVHD) reaction, said method comprising the steps of:

a) selecting an immunodominant minor histocompatibility antigen (MiHA) expressed by target cells of said recipient but not expressed by any of the donor's cells, wherein said immunodominant MiHA is expressed by the recipient's target cells at a level of at least about 100 copies per cell;

b) isolating T-cells from said donor; and

c) • positively selecting, from the T-cells isolated at step b), T-cells that specifically recognize the immunodominant MiHA of step a); and/or

- eliminating, from the T-cells isolated at step b), T-cells reacting against any immunodominant minor histocompatibility antigen(s) other than the immunodominant MiHA of step a);

whereby the T-cells selected/not eliminated at step c) are capable of being transferred into a compatible recipient without causing him a graft-versus-host disease (GVHD) reaction.

14. The method of claim 13, wherein step c) is performed so that 100% of the T-cells selected/not eliminated specifically recognize said immunodominant MiHA.

15. The method of claim 13 or 14, further comprising the step of priming *in vitro* the T-cells selected/not eliminated at step c) against said MiHA such that, when transferred into said recipient, the primed T-cells eliminate the target cells of the recipient.

16. The method of any one of claims 13 to 15, further comprising the step of genetically modifying at least some of the T-cells isolated from said donor such that said modified T-cells produce at least one lymphokine selected from the group consisting of IL-2, IL-4, IL-6, IL-7 and IL-15.

17. The method of any one of claims 13 to 16, wherein said T-cells are CD4+ or CD8+ T-cells and wherein the recipient's target cells are selected from the group consisting of cancerous cells, viral infected cells, and abnormal cells at a specific phase of the cell cycle or at a specific differentiation stage.

18. A method for selecting CD8+ T-cells from a hematopoietic cancer-free donor which are capable of eliminating hematopoietic cancerous cells when transferred into a compatible hematopoietic cancerous recipient without causing the recipient a graft-versus-host disease (GVHD) reaction, said method comprising the steps of:

a) selecting a ubiquitous and immunodominant minor histocompatibility antigen (MiHA) expressed by hematopoietic cells of said recipient but not expressed by any of the cells of said donor;

b) isolating CD8+ T-cells from said donor;

c)
- positively selecting, from the T-cells isolated at step b), the CD8+ T-cells that specifically recognize the MiHA of step a); and/or
- eliminating, from the T-cells isolated at step b), the CD8+ T-cells reacting against immunodominant minor histocompatibility antigen(s) other than the MiHA of step a);

and

d) priming *in vitro* the CD8+ T-cells selected/not eliminated at step c) against the MiHA of step a);

whereby, the CD8+ T-cells primed at step d) are capable of being transferred into a compatible hematopoietic cancerous recipient for eliminating hematopoietic cancerous cells of said recipient without causing him a graft-versus-host disease (GVHD) reaction.

19. The method of claim 18, wherein the donor and the recipient are humans.

20. In a method for eliminating, in a recipient, target cells expressing a selected immunodominant minor histocompatibility antigen (MiHA), without causing the recipient a graft-versus-host-disease reaction; the method comprising the steps of:

- isolating T-cells from a compatible donor;
- transferring said T-cells to the recipient; and
- priming the donor's T-cells against said immunodominant minor histocompatibility antigen (MiHA);

the improvement wherein at least one of the following conditions is satisfied:

- 100% of the T-cells transferred from said donor into said recipient specifically recognize said immunodominant MiHA;
- said immunodominant MiHA is ubiquitous and expressed by all the cells of the recipient, but is not expressed by any of the cells of said donor.

21. The method of claim 20, wherein said T-cells are CD4+ or CD8+ T-cells and wherein the recipient's target cells are selected from the group consisting of cancerous cells, viral infected cells, and abnormal cells at a specific phase of the cell cycle or at a specific differentiation stage.

22. The method of claim 20 or 21, wherein the donor and the recipient are humans.

23. In a method for treating an hematopoietic cancer wherein CD8+ T-cells from a hematopoietic cancer-free donor are isolated and transferred into a compatible hematopoietic cancerous recipient; the improvement wherein the CD8+ T-cells from said donor are primed against a ubiquitous and immunodominant minor histocompatibility antigen (MiHA) expressed by all the recipient's cells but not expressed by any of the donor's cells.

24. The method of claim 23, wherein the CD8+ T-cells from said donor are primed *in vitro* against said MiHA prior to their transfer into the recipient.

25. The method of claim 23 or 24, wherein levels of expression for said MiHA by non-hematopoietic cells of said recipient are not greater than about 10% of levels of expression for said MiHA by hematopoietic cells of said recipient.

26. The method of any one of claims 23 to 25, wherein said ubiquitous immunodominant MiHA is expressed by the hematopoietic cells of said recipient at a level of at least about 100 copies per cell.

27. The method of any one of claims 23 to 26, wherein, prior to said transfer, all CD8+ T-cells reacting against any immunodominant minor histocompatibility antigen(s) other than said ubiquitous immunodominant MiHA are eliminated.

28. The method of any one of claims 23 to 27, wherein prior to said transfer, all CD8+ T-cells reacting against any minor histocompatibility antigen(s) other than

said ubiquitous immunodominant MiHA are eliminated such that 100% of the CD8+ T-cells transferred from said donor into said recipient specifically recognize said ubiquitous immunodominant MiHA.

29. The method of any one of claims 23 to 28, further comprising at least one step selected from the steps of:

a) positively selecting isolated CD8+ T-cells that specifically recognize said MiHA and transferring solely said positively selected CD8+ T-cells into said recipient; and

b) eliminating CD8+ T-cells reacting against immunodominant minor histocompatibility antigen(s) other than said recipient's MiHA prior to transferring the donor's CD8+ T-cells into said recipient.

30. The method of claim 29, wherein said CD8+ T-cells are positively selected and/or eliminated using a method selected from the group consisting of flow cytometry and *in vitro* expansion of CD8+ T-cells specifically recognizing said ubiquitous immunodominant MiHA.

31. The method of any one of claims 23 to 30, further comprising the step of preventing activation induced cell death (AICD) of the CD8+ T-cells transferred into the recipient.

32. The method of any one of claims 23 to 31, wherein the donor and the recipient are humans.

33. Use of a population of T-cells according to any one of claims 1 to 9 and 11, and/or of a therapeutic physiological solution according to claim 10 or 12, for eliminating hematopoietic cancerous cells into a human recipient.

34. Use of a population of T-cells according to any one of claims 1 to 9 and 11, and/or of a therapeutic physiological solution according to claim 10 or 12, for the

preparation of a pharmaceutical composition for eliminating hematopoietic cancerous cells into a human recipient.

A) Unprimed Recipients

Recipient	Antigenic disparities between recipient and EL4 cells	Symbol
B6	6 TAAs	○
B10	6 TAAs + H9^{-a} MiHA	◆
B10.H7^{b}	6 TAAs + H9^{-a} and B6^{dom1} MiHAs	■
C3H/HeJ	full H2^{b} haplotype	▲

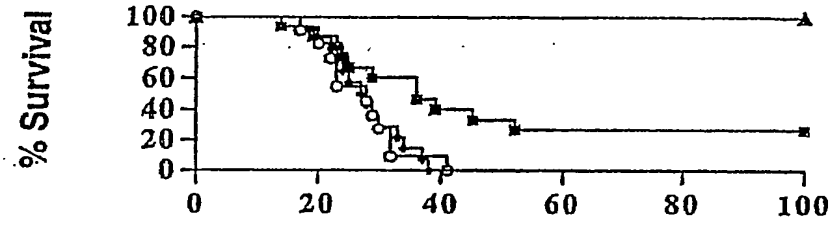


FIG. 1A

B) Primed Recipients

Recipient	Priming	Antigens present in both the priming inoculum and EL4 cells	Symbol
B10	EL4 cells	6 TAAs + H9^{-a} MiHA	◆
B10.H7^{b}	B10 cells	B6^{dom1} MiHA	△
B10.H7^{b}	LP cells	B6^{dom1} MiHA	■
B10.H7^{b}	B6^{dom1} peptide	B6^{dom1} MiHA	○

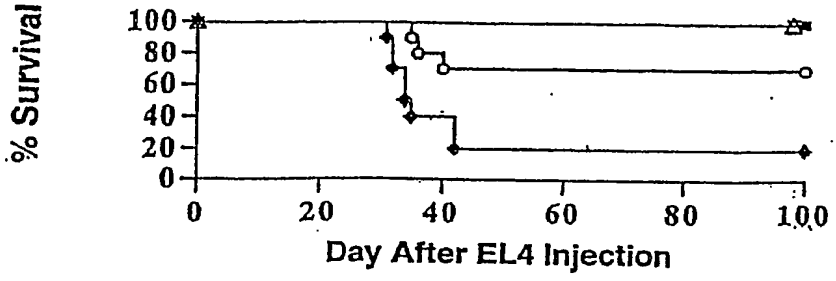
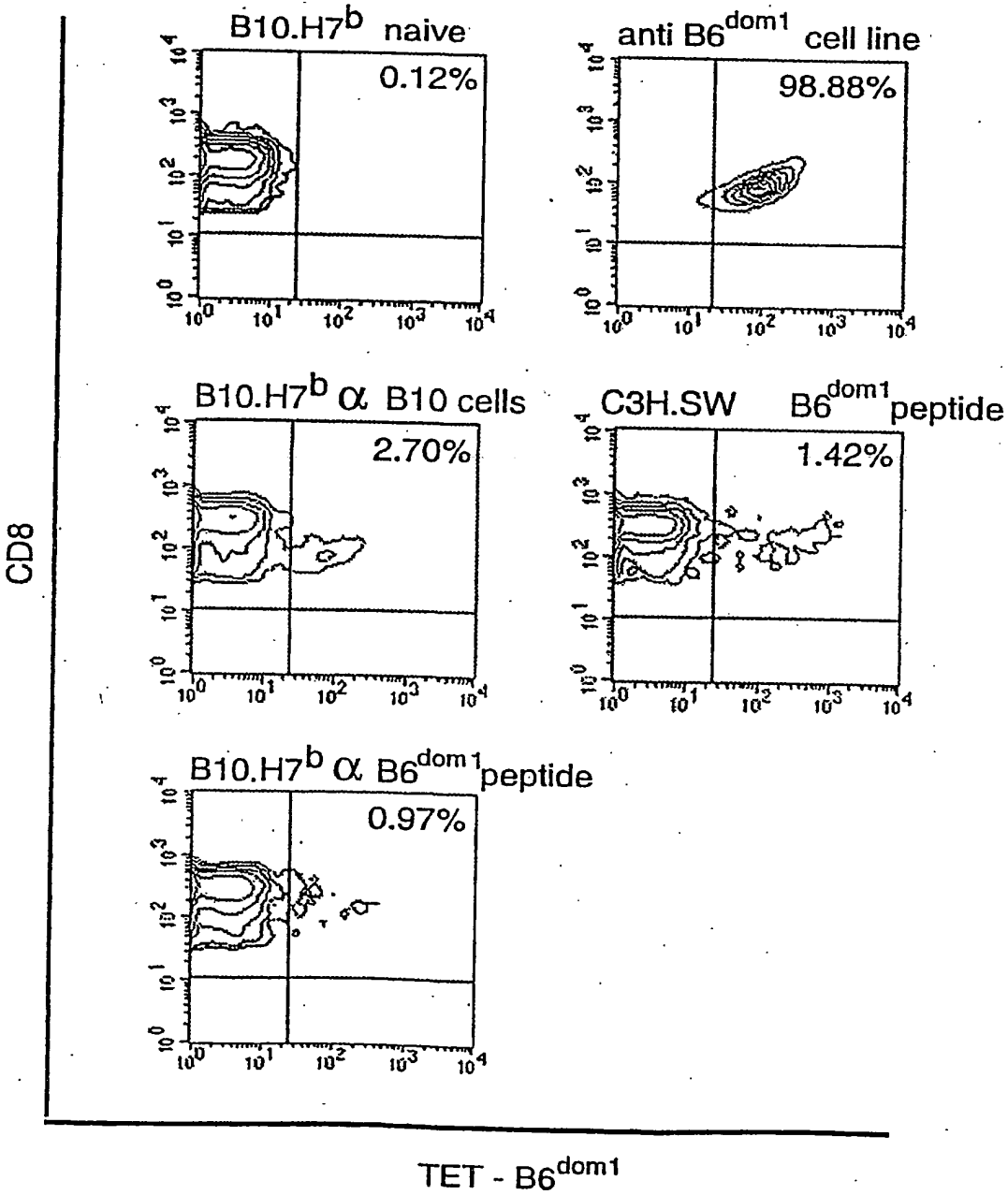


FIG. 1B

FIG. 2



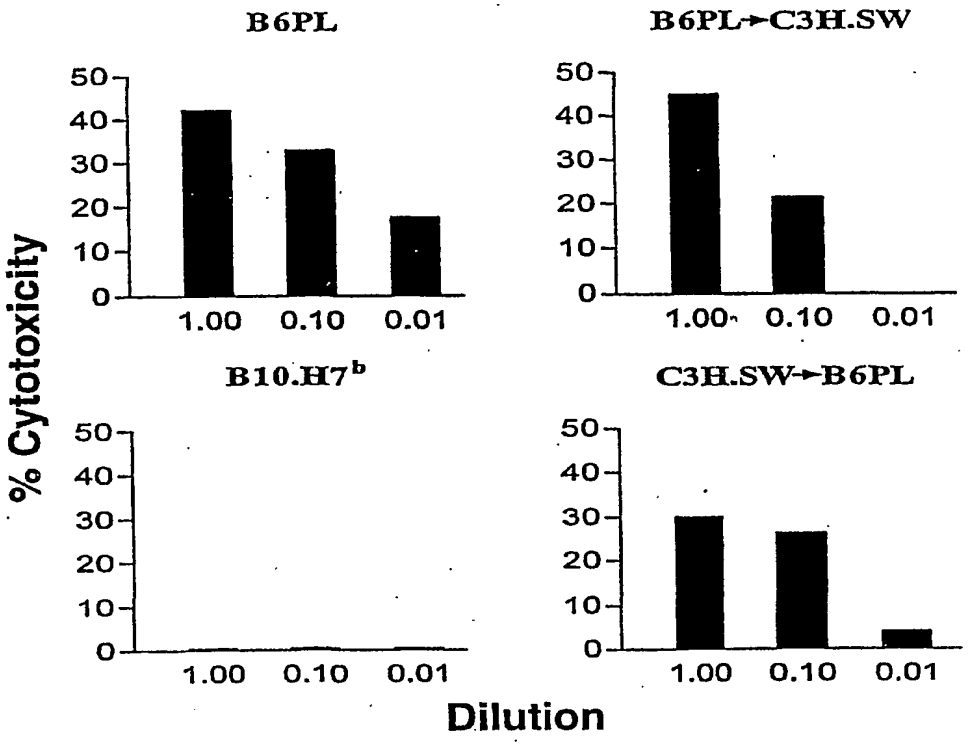


FIG. 3

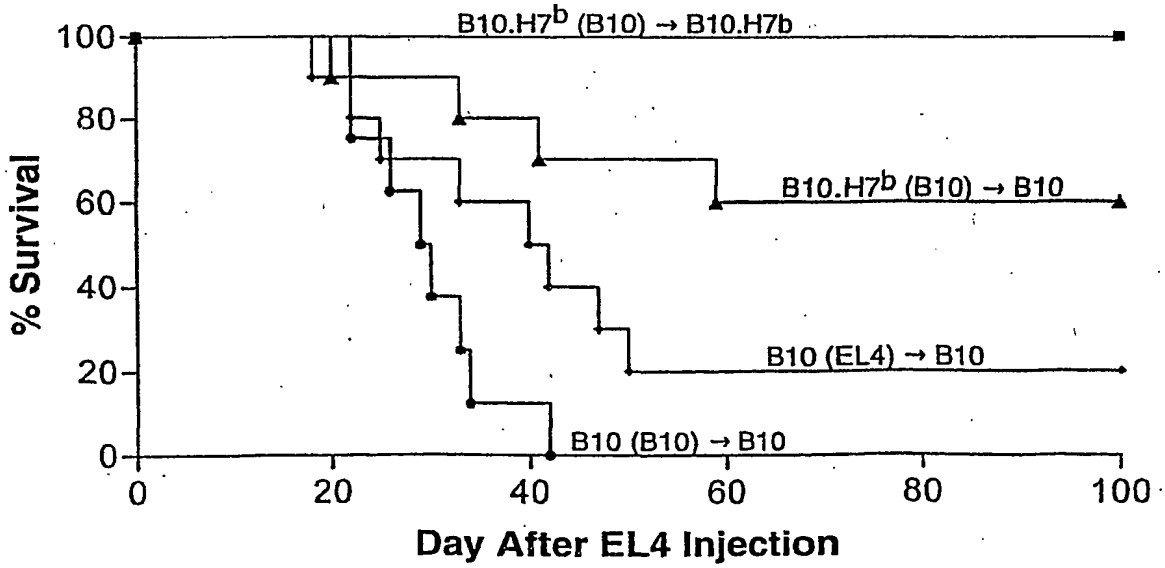


FIG. 4

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Declaration under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for all designations*

Published:
— *with international search report*

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27 December 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/036750 A3

(54) Title: T-CELLS SPECIFICALLY RECOGNIZING MINOR HISTOCOMPATIBILITY ANTIGEN(S) AND USES THEREOF FOR ELIMINATING TARGET CELLS

(57) Abstract: This invention relates to T-cells that specifically recognize minor histocompatibility antigen(s), methods for selecting these T-cells and uses thereof for eliminating target cells, and more particularly for eliminating hematopoietic cancerous cells. The invention is based on 1) the priming of T-cells specifically reacting against a selected immunodominant ubiquitous MiHA that is expressed by target cells and by non-target cells; and also 2) the selection of a 100 % purified population of T-cells that specifically react against an immunodominant minor histocompatibility antigen which is ubiquitously expressed by the recipient's cells or selectively expressed by specific recipient's target cells only. The major advantage of the invention is that the T-cells used therein can be transferred from a donor to a compatible recipient without causing to the latter a graft-versus-host disease (GVHD) reaction.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 01/01477

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/08 C12N5/10 A61K39/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, EPO-Internal, BIOSIS, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MUTIS TUNA ET AL: "Feasibility of immunotherapy of relapsed leukemia with ex vivo-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens." BLOOD, vol. 93, no. 7, 1 April 1999 (1999-04-01), pages 2336-2341, XP002210313 ISSN: 0006-4971	1-3, 7-12, 20-26, 32-34
Y	the whole document	13-15, 17-19, 27,28

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 August 2002	03/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Noë, V

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 01/01477

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 020 519 A (INTROGENE BV) 19 July 2000 (2000-07-19) abstract page 2, paragraph 5 - paragraph 8 page 3, paragraph 21 -page 4, paragraph 28 page 8, paragraph 60	1-5, 7-12, 20-26, 32-34
Y		13-19, 27,28
Y	DIJK VAN A M C ET AL: "SELECTIVE DEPLETION OF MAJOR AND MINO HISTOCOMPATIBILITY ANTIGEN REACTIVE T CELLS: TOWARDS PREVENTION OF ACUTE GRAFT-VERSUS-HOST DISEASE" BRITISH JOURNAL OF HAEMATOLOGY, OXFORD, GB, vol. 1, no. 107, October 1999 (1999-10), pages 169-175, XP001074673 ISSN: 0007-1048 the whole document	13-19, 27,28
A	PERREAULT C ET AL: "Immunodominant minor histocompatibility antigens: the major ones" IMMUNOLOGY TODAY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 19, no. 2, 1 February 1998 (1998-02-01), pages 69-74, XP004107029 ISSN: 0167-5699	
P,X	FONTAINE P ET AL: "Adoptive transfer of minor histocompatibility antigen-specific T lymphocytes eradicates leukemia cells without causing graft-versus-host disease." NATURE MEDICINE. UNITED STATES JUL 2001, vol. 7, no. 7, July 2001 (2001-07), pages 789-794, XP002210314 ISSN: 1078-8956 the whole document	1-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 01/01477

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 20-33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int...nal Application No
PCT/CA 01/01477

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1020519 A	19-07-2000	EP 1020519 A1	19-07-2000
		AU 2332000 A	01-08-2000
		EP 1141280 A1	10-10-2001
		WO 0042181 A1	20-07-2000

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